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14. ABSTRACT

Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of this disease. The objective of our research is to determine how nutritional compounds genistein, betasitosterol (SIT), and omega-6 fatty acids (FA) function as modulators of PCa. In the third and final year of this fellowship, the fellow joined the National Cancer Institute's Cancer Prevention Fellowship Program and has continued working on the DOD-PCRP Postdoctoral Fellowship project with 30% effort (with knowledge and approval from the DOD-PCRP). During this year, we finished printing our custom microarray as well as optimized the hybridization protocol. We are currently performing expression analysis using the microarrays for both the *in vitro* and *in vivo* experiments using well established cell lines. Finally, we have initiated xenograft *in vivo* experiments with a novel PCa model that was developed recently in a collaborating laboratory.

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Introduction

Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of this disease. The objective of our research is to determine how nutritional compounds genistein, betasitosterol (SIT), and omega-6 fatty acids (FA) function as modulators of PCa. These three compounds belong to three distinct classes of dietary components, specifically isoflavonoid (genistein), phytosterol (SIT), and FA (omega-6 FA). Each class of compound could potentially modulate PCa in different ways and previous investigations have indicated that all three compounds may affect PCa in a dose-dependent fashion. We will determine differences in gene expression profiles at a range of doses for each compound on *in vitro* models. We will then test up to two doses on *in vivo* models to account for the complexity of tumor microenvironments.

In the first year, we began to develop the technical tools with which to investigate gene expression patterns that are modulated by the 3 dietary compounds. In the second year of study, we finished preparation of 355 cDNAs for fabricating the custom microarray for analysis of gene expression. In addition, we completed *in vitro* and *in vivo* experiments using well established PCa cell lines and the three dietary compounds in varying concentrations. In the third year, the fellow joined the National Cancer Institute's Cancer Prevention Fellowship Program and has continued working on the DOD-PCRP Postdoctoral Fellowship project with 30% effort (with knowledge and approval from the DOD-PCRP). During this past year, we finished printing our custom microarray as well as optimized the hybridization protocol. We are currently performing expression analysis using the microarrays for both the *in vitro* and *in vivo* experiments using well established cell lines. Finally, we have initiated xenograft *in vivo* experiments with a novel PCa model that was developed recently in a collaborating laboratory.

Here, we report our progress as it relates to the approved Statement of Work:

Task 1: To delineate the gene expression patterns of PCa *in vitro* of three nutritional compounds.

1.1 Obtain cDNAs for microarrays

From year 2, 344 cDNA clones from either in-house resources or ATCC have been prepared for our custom microarray. Genes chosen were tyrosine kinases or involved in cellular pathways with emphasis on androgen receptor (AR) and apoptosis pathways. Also included in our gene list are 3 control genes (GAPD, ACTB, and MBP) and other controls (Human Cot-1, Mouse Cot-1, Salmon Sperm DNA, and male human genomic DNA) for a total of 352 features.

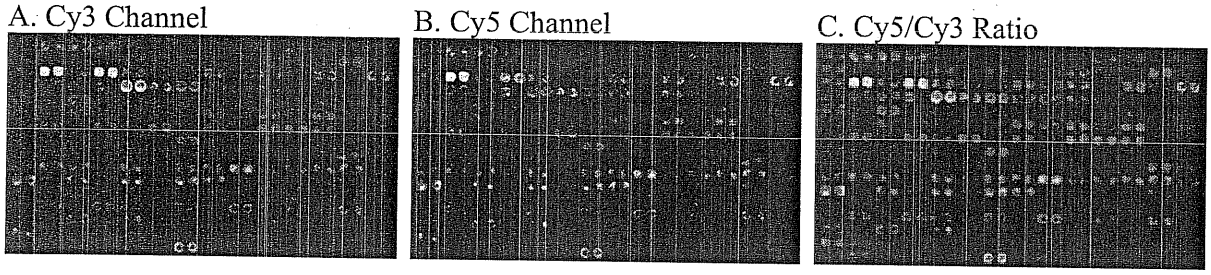
1.2 Fabricate arrays and optimize protocols

We have successfully fabricated our custom microarray with the above mentioned 352 features (in duplicate) (Table 1). For hybridization, we evaluated incorporating Cyanine 3- (Cy3) and Cyanine 5-dUTPs (Cy5) (Amersham) in the control and test samples by 2 methods: direct incorporation during reverse transcription polymerase chain reaction (RT-PCR) and during random priming (RP) after RT-PCR. Hybridization using labeled cDNA resulted in weak signals in the Cy3 probes and little or no signals in the Cy5 probes. Hybridization using RP probes resulted in much stronger signals in both Cy3 and Cy5 channels. A representative example of an array hybridization is shown in Figure 1. For subsequent array experiments, RP probe labeling will be utilized after RT-PCR of total mRNA from each sample.

Table 1: Map of the custom microarray printing

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	MMP1	MMP1	KLK2	KLK2	AKT1	AKT1	BRCA1	BRCA1	TNFSF12	TNFSF12	CDC25A	CDC25A	DFFA	DFFA	MDM2	MDM2	CASP4	CASP4	RB1	RB1	TNFSF6	TNFSF6	ATM	ATM	D4GDI	D4GDI	RAD53	RAD53
2	CAFP6	CAFP6	CDK4	CDK4	FADD	FADD	CDKN1B	CDKN1B	EIF2S1	EIF2S1	TP53	TP53	BCL2L1	BCL2L1	CDKN1A	CDKN1A	BAD	BAD	PRKCD	PRKCD	USACYP	USACYP	CDC2	CDC2	ESRRA	ESRRA	CDC25A	CDC25A
3	MXI1	MXI1	CDKN1A	CDKN1A	ALOX5AP	ALOX5AP	CDKN2A	CDKN2A	SOS1	SOS1	DIABLO	DIABLO	IL2RA	IL2RA	FADD	FADD	BCAR3	BCAR3	GADD45A	GADD45A	PPARA	PPARA	KRT18	KRT18	ALOX15B	ALOX15B	LMNA	LMNA
4	HRAS	HRAS	PDPK1	PDPK1	EBBP	EBBP	PLK1	PLK1	PRKCA	PRKCA	PRSS25	PRSS25	CASP9	CASP9	MBP	MBP	CASP2	CASP2	GAPD	GAPD	CFLAR	CFLAR	PLAUR	PLAUR	TRAF1	TRAF1	PLAU	PLAU
5	DEDD	DEDD	NME4	NME4	GAS2	GAS2	KISS1	KISS1	TRADD	TRADD	KAI1	KAI1	CASP1	CASP1	PAI2	PAI2	TNFRSF10B	TNFRSF10B	SERPINE1	SERPINE1	ITGA2	ITGA2	TIMP1	TIMP1	ITGB5	ITGB5	ETS2	ETS2
6	ITGA1	ITGA1	RASA1	RASA1	VIM	VIM	SFN	SFN	SRF	SRF	TNFRSF10D	TNFRSF10D	EBAG9	EBAG9	TNFSF6	TNFSF6	TAB1	TAB1	TP53	TP53	POV1	POV1	YWHAQ	YWHAQ	HPGD	HPGD	ACTA1	ACTA1
7	IGFBP3	IGFBP3	CASP4	CASP4	MADH4	MADH4	CYCS	CYCS	CTNNB1	CTNNB1	FGF7	FGF7	DEDD	DEDD	DFFA	DFFA	ESR1	ESR1	TNFRSF10C	TNFRSF10C	MAP2K2	MAP2K2	BAG4	BAG4	CD44	CD44	RAF1	RAF1
8	ITGA3	ITGA3	PIK3CB	PIK3CB	ICAM1	ICAM1	MAP2K1	MAP2K1	ITGAV	ITGAV	PIK3R1	PIK3R1	MICA	MICA	FGFR2	FGFR2	ITGA5	ITGA5	TEK	TEK	THBS1	THBS1	PDGFRA	PDGFRA	COL18A1	COL18A1	ABL1	ABL1
9	THBS2	THBS2	ERBB2	ERBB2	EGF	EGF	SRC	SRC	VEGF	VEGF	IGF1R	IGF1R	HGF	HGF	FER	FER	FGR	FGR	CASP7	CASP7	VDR	VDR	TNFRSF6	TNFRSF6	ESR2	ESR2	TNFRSF6B	TNFRSF6B
10	BAD	BAD	ABCC6	ABCC6	NUMA1	NUMA1	ALOX12	ALOX12	ALOX12	ALOX12	IKBKG	IKBKG	BMX	BMX	MAP3K14	MAP3K14	ALOX5	ALOX5	MAP3K5	MAP3K5	TERT	TERT	NFKB1B	NFKB1B	C10orf10	C10orf10	SPTAN1	SPTAN1
11	ALDH1	ALDH1	YWHAG	YWHAG	BIRC5	BIRC5	ITGA6	ITGA6	ANGPT2	ANGPT2	KIT	KIT	ANGPT1	ANGPT1	PTK7	PTK7	CDH1	CDH1	TYK2	TYK2	IGF1	IGF1	MERTK	MERTK	RPA3	RPA3	DDR	DDR
12	PCNA	PCNA	FGFR3	FGFR3	CCND1	CCND1	TXK	TXK	CDC37	CDC37	RYK	RYK	MTA1	MTA1	HCK	HCK	NFKBIA	NFKBIA	EPHB3	EPHB3	MAPKAPK2	MAPKAPK2	LYN	LYN	GSTP1	GSTP1	EPHA1	EPHA1
13	SERPINB5	SERPINB5	ACTB	ACTB	STAT1	STAT1	ABL2	ABL2	AATK	AATK	EPHA7	EPHA7	HNF3A	HNF3A	BLK	BLK	EPHB6	EPHB6	BIRC2	BIRC2	FGF2	FGF2	TNFRSF6	TNFRSF6	PTK2	PTK2	PXN	PXN
14	CSK	CSK	CRADD	CRADD	ADPRT	ADPRT	CASP7	CASP7	ATM	ATM	CASP10	CASP10	BCL2	BCL2	BIRC4	BIRC4	BCL2A1	BCL2A1	CASP3	CASP3	MYBL2	MYBL2	LCK	LCK	NR1D1	NR1D1	NTRK2	NTRK2
15	RAP1A	RAP1A	EPHB1	EPHB1	KLK3	KLK3	MST1R	MST1R	ODC1	ODC1	SRM	SRM	HEVIN	HEVIN	MET	MET	HPN	HPN	NTRK3	NTRK3	LIM	LIM	EPHB2	EPHB2	FOLH1	FOLH1	ERBB3	ERBB3
16	FKBP5	FKBP5	PTK9	PTK9	ADAM9	ADAM9	YES1	YES1	KCNMB1	KCNMB1	TIE	TIE	BCL2L11	BCL2L11	TRAF2	TRAF2	BIRC2	BIRC2	TNFSF10	TNFSF10	BIRC3	BIRC3	TNFRSF6	TNFRSF6	BIRC4	BIRC4	TLN1	TLN1
17	CASP10	CASP10	PARP	PARP	CASP3	CASP3	TNFRSF6B	TNFRSF6B	CDKN1B	CDKN1B	BCL2A1	BCL2A1	CRADD	CRADD	TNFSF10A	TNFSF10A	CREB1	CREB1	DFFB	DFFB	DFFB	DFFB	PTK2B	PTK2B	LRDD	LRDD	GZMB	GZMB
18	MAPK10	MAPK10	NTRK1	NTRK1	NCOA4	NCOA4	NTRKR1	NTRKR1	MDH1	MDH1	ACK	ACK	TUBA3	TUBA3	INSR	INSR	RNF14	RNF14	TEC	TEC	SRD5A2	SRD5A2	JAK1	JAK1	AKR1B1	AKR1B1	PTK6	PTK6
19	CHD4	CHD4	EPHA2	EPHA2	GAGEB1	GAGEB1	BTK	BTK	CSNK2B	CSNK2B	CSF1R	CSF1R	AR	AR	KDR	KDR	TGM4	TGM4	LMTK2	LMTK2	DBI	DBI	ROS1	ROS1	MYC	MYC	PPARG	PPARG
20	PMAIP1	PMAIP1	MMP9	MMP9	TNF	TNF	BID	BID	TNFRSF10A	TNFRSF10A	ESRRG	ESRRG	TNFRSF10B	TNFRSF10B	FLT3	FLT3	TNFRSF21	TNFRSF21	ANXA5	ANXA5	TNFSF10	TNFSF10	MADD	MADD	TRAF2	TRAF2	PRF1	PRF1
21	YWHAE	YWHAE	FLT1	FLT1	ARHGDIB	ARHGDIB	ITGA2B	ITGA2B	BAK1	BAK1	LMNA	LMNA	BAX	BAX	ITGB3	ITGB3	PPAP2B	PPAP2B	ITK	ITK	JTB	JTB	FLT4	FLT4	CLU	CLU	EPHA3	EPHA3
22	FAT	FAT	SYK	SYK	SFRP4	SFRP4	FRK	FRK	BCRP1	BCRP1	DKFZp434C1418	DKFZp434C1418	PSCA	PSCA	ROR2	ROR2	PPAP2A	PPAP2A	EPHA4	EPHA4	C7	C7	ZAP70	ZAP70	C2ORF1	C2ORF1	EPHA5	EPHA5
23	RAB5A	RAB5A	ALK	ALK	ANXA7	ANXA7	FGFR4	FGFR4	BID	BID	PLAB	PLAB	BIK	BIK	CDKN2A	CDKN2A	BIRC5	BIRC5	HuGeNMale	HuGeNMale	BIRC7	BIRC7	HuCot1	HuCot1	CASP2	CASP2	MsGeNMale	MsGeNMale
24	CASP6	CASP6	MSCot1	MSCot1	CASP8	CASP8	SSGeNDNA	SSGeNDNA	CASP9	CASP9	SSGeNDNA	SSGeNDNA																
25	CSTB	CSTB	PRKCB1	PRKCB1	ADM	ADM	MYB	MYB	SREBF1	SREBF1	N33	N33	SHBG	SHBG	IGF1	IGF1	TMPRSS2	TMPRSS2	KRAS2	KRAS2	DKFZP434D146	DKFZP434D146	HPR6.6	HPR6.6	IMPDH2	IMPDH2	IL2RB	IL2RB
26	TGFB1I1	TGFB1I1	NRAS	NRAS	CHEK2	CHEK2	HNF3G	HNF3G	ACPP	ACPP	PDGFA	PDGFA	LDHA	LDHA	MADH2	MADH2	FKBP8	FKBP8	CCNT1	CCNT1								

Figure 1: Representative hybridization patterns of RP labeled cDNAs in Cy3 channel (**A**), Cy5 channel (**B**) and Cy5/Cy3 ratio (**C**).



1.3 Grow cells under test conditions, isolate RNA, RT-PCR, label probes

We completed the cell culture experiments of this task in year 2. However, we were unable to replicated experiments using the test compound SIT due to difficulties in consistently dissolving SIT into solution with its appropriate vehicle (Cyclodextrin; Sigma). Because we successfully tested this compound in our *in vivo* xenograft model pilot study (Task 2), we have decided to delay the *in vitro* experiments using SIT until we are able have appropriate resources to reliably perform the experiments.

1.4 Perform microarray experiments, analyze data with standard statistical programs and Perform multicolor spectral transcript analysis and analyze data with standard statistical programs

Due to the time needed to optimize the hybridization protocol, we are currently in the process of performing array experiments and analyzing the expression patterns from RNA isolated from the *in vitro* experiments. See Figure 2 under Task 2 for examples of array hybridizations. The multicolor spectral transcript analysis will need to be delayed until all array experiments have been completed.

Task 2: To determine if and how three select dietary components modulate the growth and gene expression of clinical PCa specimens in vivo.

2.1 Pilot study to investigate timing of nutrient supplementation in SCID mice

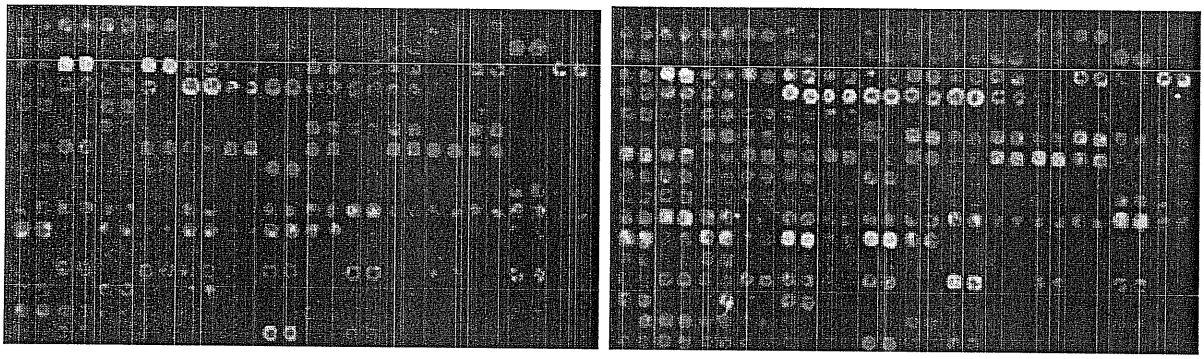
In parallel with the *in vitro* experiments mentioned in Task 1, we performed *in vivo* experiments using xenograft technique on SCID mice at the co-mentor's laboratory using the same cell lines mentioned above in year 2.

2.2 Analyze tumors from pilot study

Similar to task 1.4, we are currently performing array experiments and analyzing the expression patterns from RNAs isolated from the *in vivo* experiments. Examples of hybridizations using RNAs from this pilot study are shown in Figure 2. Likewise, the multicolor spectral transcript analysis will need to be delayed until all array experiments have been completed.

Figure 2: Representative hybridization patterns of CAFTD04 derived tumors grown in SCID mice fed with control diet (**A**) and Genistein supplemented diet (**B**). All test probes are labeled in Cy3 (green) and reference probes (generated from CAFTD04 derived tumors grown in SCID mice fed with control diet) are labeled in Cy5 (red).

A. **B.**



2.3 Grow 6 tumors in 21 SCID mice (2 tumors per mice) that ingest one of 7 test diets, harvest tissues, prepare tissue sections and RNAs, RT-PCR, and make probes. Test diets include control and each compound at one of 2 concentration

For this task, we found it difficult to obtain clinical samples that were large enough to be implanted into the number of mice that was initially proposed. To circumvent this problem, we decided to use a newly generated PCa model that was developed by a collaborator (Wang et al., submitted) as mentioned in the previous report. Briefly, pieces of PCa tissue from a patient were grafted into the subrenal capsule site of testosterone-supplemented male SCID mice. After 5 serial transplantations, the tissues were transferred into mouse prostates. A metastatic tumor line generated from lymph nodes, designated PCa1-met, had few chromosomal alterations, as indicated by Spectral Karyotyping. Orthotopic grafting of PCa1-met in 47 hosts led in all cases to metastases to multiple organs (lymph nodes, lung, liver, kidney, spleen and, notably, bone). Histopathological analysis showed strong similarity between orthotopic grafts and their metastases which were of human origin as indicated by immunostaining using antibodies against human mitochondria, androgen receptor, prostate-specific antigen, pan-cytokeratin, p63, cytokeratin-8 and Ki-67. This model is unique due to the fact that the xenograft implants metastasized to regions of the mouse that paralleled the locations of clinical PCa metastases (most notably lung, liver and bone) in humans. In addition, the resulting cell lines contain few cytogenetic aberrations, similar to clinical PCa and unlike most well established cell lines (i.e., PC3, LNCaP, and DU145).

When we implemented the experiments using this new model, however, the SCID mice were adversely affected in unanticipated ways. At the same time, our collaborators were having similar problems. All animal experiments were stopped so that the issue can be resolved without harm to any more animals. Recent experiments as communicated by our collaborators showed that the murine stroma, which was contained within the tumors that are regrafted, harbored a mouse virus that infected the host SCID mice. The contaminated tumor lines have since been discarded and several un-infected tumor grafts have been established. We are currently in the process of using the new and un-infected tumor lines for the purpose of this study.

2.4 Perform microarray experiments, analyze data with standard statistical programs

Due to the problem described above, we are currently in the process of performing the *in vivo* experiments using the newly established PCa model. Once these experiments are complete, we will analyze the expression profiles using our custom arrays.

Key Research Accomplishments for Year 2

- Fabricated a custom microarray with 352 features in duplicate.
- Optimized microarray hybridization conditions.
- Began microarray expression profiling of RNAs from *in vitro* experiments with established PCa cell lines grown in control and test media containing different concentrations of test nutritional supplements.
- Began microarray expression profiling of RNAs from *in vivo* experiments in the co-mentor's laboratory with xenografts of well established PCa cell lines in SCID mice whose diets are supplemented with various test compounds.
- Initiated *in vivo* experiments in co-mentor's laboratory with xenografts of the newly developed and un-infected PCa tumor lines in SCID mice whose diets are supplemented with various test compounds.

Reportable Outcomes

- Became a National Cancer Institute Cancer Prevention Fellow (2005-2008)
    - Beginning in July 2005, the fellow began a 1 year academic program to obtain a Masters in Public Health with an emphasis on Epidemiology at the University of California, Berkeley to supplement the fellow's training in the field of cancer prevention. Degree is expected to be conferred in May 2006.
    - Beginning in June 2006, the fellow will begin a 2 year research program at the National Cancer Institute in Bethesda, MD, which will focus on cancer prevention.

Conclusion

We have used the third year of this fellowship to fabricate our custom microarray with 352 features (in duplicate) for expression profiling of cells grown *in vitro* and *in vivo* under different dietary supplement conditions including none, genistein, SIT, and omega-6 FA. In addition, this year has been used to troubleshoot many unexpected issues such as microarray probe generation as well as viral infection of the new PCa tumor line models. Both major issues have been resolved. Microarray analysis for task 1 and the pilot study for task 2 are underway. Additionally, the *in vivo* xenograft model using the new PCa tumor lines have been initiated and expression profiling of the resulting tumors will be performed once tumors are harvested from co-mentor's laboratory.

Publications

None: pending final data analyses.